Harmful Algae 39 (2014) 191–201

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

First evidence of cell deformation occurrence during a *Dinophysis* bloom along the shores of the Gulf of Tunis (SW Mediterranean Sea)

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ARTICLE INFO

Article history:
Received 23 May 2014
Received in revised form 29 July 2014
Accepted 29 July 2014
Available online

Keywords:
Dinophysis
Deformations
Diel cycles
Seasons
Mediterranean

ABSTRACT

Never before observed or cited in *Dinophysis* studies, deformations in *Dinophysis acuminata* and *Dinophysis sacculus* are reported throughout their cellular division phases (cytokinesis, and sulcal list regeneration) in 5 *in situ* cell cycle studies in the Punic harbors of Carthage (northern Tunisia). Two types of deformation were observed: invaginations in the ventral and dorsal margin and protuberances at the base of the left sulcal list. No virus or bacteria were detected with Syber green stain. *In situ* division rates (μ) varied among seasons and stations for the same species. *D. acuminata* exhibited moderate (0.22 day^{-1}) to high (0.68 day^{-1}) μ rates which were however very low (0.02–0.17 day^{-1}) for *D. sacculus* in autumn and moderate (0.21–0.35 day^{-1}) in late spring. In 2009 the seasonal distribution of *Dinophysis* indicates maximum *Dinophysis cf. ovum* abundance in March and a high number of *D. acuminata* in early June, while in 2010 maximum abundance of the same species was found in mid-June.

Molecular and genetic studies and staining with specific fluorescent strains should be addressed to hopefully explain these *Dinophysis* cell deformations during their *in situ* division.

1. Introduction

The main gaps in knowledge concerning the biology and population dynamics of the genus of *Dinophysis* were reviewed from 1978 to 1998 (Yasumoto et al., 1978, 1980; Hallegraeff and Lucas, 1988; Lassus and Bardouil, 1991; Bravo et al., 1995; Steidinger and Tangen, 1996; Maestrini, 1998). Based on the different results of these studies, many new observations have been recorded since 2001, such as small and intermediate forms (Reguera and González-Gil, 2001), cell-cycle stages (Reguera et al., 2003) and feeding behaviour (Park et al., 2006). Recently, extensive progress has been made thanks to new sampling strategies (GEOHAB, 2008), the application of molecular and analytical techniques and, finally, the successful establishment of mixotrophic cultures of *Dinophysis* fed with the ciliate *Mesodinium rubrum* (Park et al., 2006). In spite of the long history of this genus (Zingone et al., 1998), many difficulties have been encountered in its taxonomic identification. Their identification is therefore principally based on the size, shape and ornamentation of the large hypothecal plates which give the cell its contour and the shape of the left sulcal lists with their three supporting ribs (Larsen and Moestrup, 1992). However, each species of *Dinophysis*, in each biogeographic region, may exhibit different sizes and shapes between the large vegetative specimens and small gamete-like cells, resulting from their polymorphic life cycles with different cell-cycle phases and feeding behaviors (Reguera and González-Gil, 2001; Reguera et al., 2003). Reguera et al. (2012) considered that the "*Dinophysis acuminata* complex", including the morphospecies described as *D. acuminata, Dinophysis sacculus* and *Dinophysis cf. ovum*, is the most common group of *Dinophysis* spp., with strains whose abundance is increasing throughout the world along coasts receiving freshwater input, and over long growing seasons (spring to autumn). In Tunisia, frequent proliferations of *D. sacculus* are associated with diarrheic toxins detected in clams and mussels in the country's northern coastal waters, as in Bizerte Lagoon (Turki et al., 2014) and in Tunis North Lagoon (Armi et al., 2011). In the Punic harbors of Carthage (northern Tunisia) every species of this group bloomed each year from 2008 to 2010 in the same periods, intriguingly exhibiting ventral and dorsal margin deformations. In this study we provide

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http://dx.doi.org/10.1016/j.hal.2014.07.017
1568-9883/

the first evidence of *Dinophysis* cell deformations observed during both one seasonal and 5 diel cycles, matching its numerical increase between 2008 and 2010 at three study stations in the Punic harbors.

2. Materials and methods

2.1. Study area

The Punic harbors of Carthage (36°50′ N 10°19′ E) are located in the southern area of the Gulf of Tunis near the Tunis North Lagoon and Radès Harbor. They are composed of two basins that cover an area of about 8 ha, connected to each other and to the Bay of Tunis by channels (Fig. 1). These coastal basins are shallow and enclosed, with an average depth of 3.20 m and only a slight exchange with the Bay of Tunis. They are considered eutrophic as they receive increasing nutrient loads from a human population in the region that has rapidly expanded since the 1990s. According to Souissi et al. (2000), the western shore of the Gulf of Tunis is generally eutrophic, as they observed a relative nutrient enrichment both here and in the harbors (Radès and La Goulette) due to urban and industrial discharges through the Rades channel and the influence of terrestrial input.

2.2. Sampling and processing

The present study is based on samples from the study of 5 diel cycles carried out essentially in early summer (June 3rd and 4th, 2008; June 15th and 16th, 2009; June 26th and 27th, 2010), and mid-autumn (November 2nd and 3rd, 2008; October 30th and 31st, 2009). Due to the shallow harbor depths, samples were collected by vertical net hauls from the entire water column using a 20-μm mesh at the three stations: station S1 (mean depth, 5.5 m), situated at the point of water exchange between the Gulf of Tunis and the north basin (larger than the southern point of connection), station S2 (3 m), corresponding to the north basin and station S3 (2.3 m), in the south basin. Temperature and salinity were simultaneously measured at each station. Chlorophyll *a* was extracted in 10 ml of 90% acetone for 24 h, in the dark at −4 °C and the extract concentration was analyzed spectrophotometrically (UV-visible spectrophotometer PU-8800).

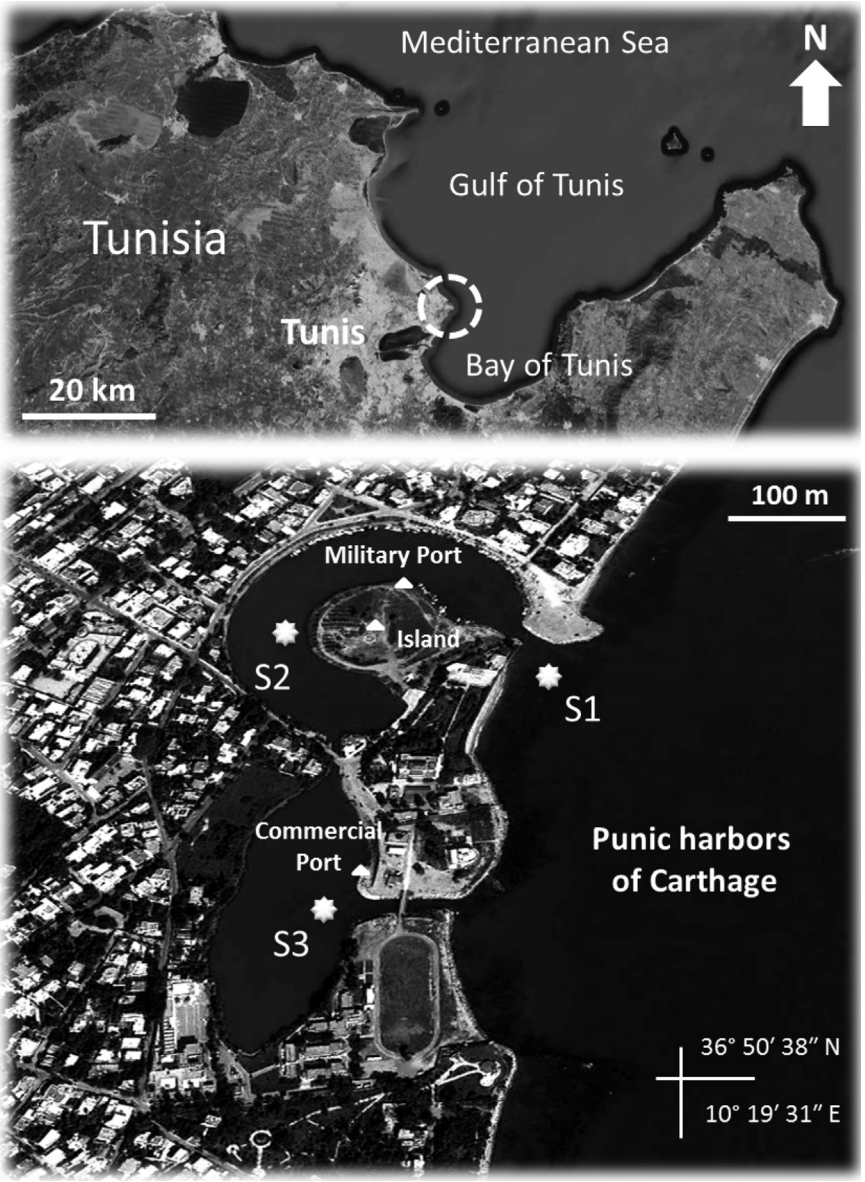

Fig. 1. Sampling site.

The vertical haul samples were further passed through a 150-μm mesh to eliminate debris and large zooplankton organisms. Both the spring and autumn cycles were conducted over periods of 22 and 26 h, with the samples being collected hourly from 11:00 (*a.m.*) to 05:00 (*a.m.*) and every 30 min from 05:00 (*a.m.*) to 11:00 (*a.m.*), corresponding to the times when cytokinesis and sulcal list regeneration can take place rapidly (Reguera et al., 2003).

Cell deformation was observed using 25-ml sedimentation chambers under an IMT2 inverted Olympus microscope. A subsample, taken from a Lugol's fixed sample, was centrifuged and the supernatant discarded. The cell pellet was rinsed with 0.45 μm filtered seawater, followed by treatment with 10% (weight/volume) sodium thiosulfate. It was then stained using SYBR Green I (35149A, Molecular probes, Invitrogen Corporation, Carlsbad, CA, USA) at 1:10,000 dilution at room temperature for 30 min. The cell pellet was observed under epifluorescence microscopy.

For the annual cycle study of *Dinophysis* spp., weekly samples were collected by vertical 20-μm mesh net hauls at each station. In all the Lugol fixed samples *Dinophysis* spp. cells were counted by the Utermöhl (1956) method using 25-ml sedimentation chambers and an IMT2 inverted Olympus microscope (the total bottom of the sedimentation chamber scanned at 200–400× magnification). Some specimens were observed under epifluorescence microscopy (Porter and Feig, 1980) using DAPI (Sigma) stain (filtration of 100 ml of sampling water through 0.22 μm pore size filter and addition of 200 μl of DAPI solution). Identification of *Dinophysis* species was performed according to descriptions established by Balech (1976a,b, 1988, 2002), Lassus and Bardouil (1991), Larsen and Moestrup (1992) and Zingone et al. (1998).

2.3. Estimates of division rates (μ)

According to Reguera et al. (2012), the potential growth rate (*sensu* Carpenter and Chang, 1988), estimated by most authors using the mitotic index approach or the specific growth rate, is μ_{gross} and results from cell division only and will be maximal under the most favorable physical conditions, availability and accessibility of resources. In contrast, the net growth rate (μ_{net}) depends on cell division, mortality due to grazing, parasitism and lyses, immigration through physical accumulation and aggregation, and emigration from physical dispersion or sinking. This mitotic index approach for estimating the growth rate has been the most widely used method in estimating the μ_{gross} of *Dinophysis* spp. in the natural population through high frequency sampling of the cell cycle. This method is based on the morphological recognition and quantification of terminal events in cells undergoing mitosis.

Estimates of *in situ* division rates were based on the mitotic index approach, calculated following the model of Carpenter and Chang (1988), using the frequency of dividing (paired) and recently divided (incomplete development of the left sulcal list) cells which were recognizable by their distinct morphology as described in Reguera et al. (2003):

$$\mu = \frac{1}{n(T_c + T_r)} \sum_{i=1}^{n} (t_s)_i \ln[1 + f_c(t_i) + f_r(t_i)] \quad (1)$$

where μ is the daily mean specific division rate, $f_c(t_i)$ is cell frequency in the cytokinetic (or paired cells with incomplete development of the left sulcal list) phase (c), and $f_r(t_i)$ is the half frequency of cells in the recently divided (missing the lower part of the left sulcal list) (r) phase in the *i*th sample. T_c and T_r are the duration of the c and r phases, considered as terminal events (*sensu* Carpenter and Chang, 1988) in the present study; n is the number of samples taken within a 24-h cycle; t_s is the sampling interval in hours.

The duration of the selected terminal events, $T_c + T_r$, has recently been defined as the division time (TD) (Carpenter and Chang, 1988) required for the cohort of cells to pass from one phase to the next. In this case, the time interval between time t_0, when the frequency of cells undergoing cytokinesis (f_c) is maximum and time t_1, when the fraction of recently divided cells f_r is maximum:

$$\frac{1}{2}(T_c + T_r) = (t_0 - t_1) \quad (2)$$

where T_c, T_r, t_1, and t_0 are calculated after fitting a fifth degree Gaussian function to the frequency data.

For the estimation of a minimum division rate during the 24-h cycle, we used the maximum frequency approach (McDuff and Chisholm, 1982). This approach supposes the possibility of recognizing all of the dividing and recently divided cells for a given day in a single sample:

$$\mu_{min} = \frac{\ln(1 + f_{max})}{\ln(1 + f_{min})} \quad (3)$$

where f_{max} is the maximum frequency (f) of dividing cells (paired plus recently divided cells) estimated following the equation:

$$f_{max} = \frac{p + I_r/2}{I_c + p + I_r/2} \quad (4)$$

where p = paired cells, I_c = fully developed individuals, and $I_r/2$ = 50% of recently divided cells (I_r).

2.4. Statistical analysis

Among the selected dominant *Dinophysis* species, we successively performed two redundancy analyses (RDA) to assess the spatial and temporal changes in species abundance, using the sampling months and sampling sites. Dinoflagellate abundance values were transformed according to the Hellinger distance prior to performing RDA. Significance of the covariates tested in the RDA was assessed through permutation tests considering 9999 permutations.

3. Results

3.1. Hydrographic parameters

During our survey, temperatures ranged from 11.6 °C in February 2008 to 30 °C in August and September 2009. There was no high temperature variation among the three stations; however, the highest temperature (30 °C) was usually recorded at S3. We recorded an increase in water temperature of 5 °C in spring and early summer and a decrease of 10 °C in mid-autumn (Table 1), coinciding with *Dinophysis* proliferations. Strong variations in salinity were recorded: 40.30 in August 2008 and 32 in December

Table 1
Average, minimum and maximum values of abiotic parameters registered at the different sampling sites. T (°C), oxygen (mg l^{-1}).

Parameters/stations	S1	S2	S3
Temperature average	20.6	20.1	20.3
Temperature min	13	11.9	10.5
Temperature max	30.2	29	30
Salinity average	35.8	36.1	35.9
Salinity min	33.6	33	33.1
Salinity max	39	40.3	39.1
pH average	7.6	7.6	7.8
pH min	3.2	4.2	3.9
pH max	10	10.3	11
Dissolved oxygen average	8.3	7.7	6.8
Dissolved oxygen min	2.4	3	3.2
Dissolved oxygen max	14.8	22.7	23

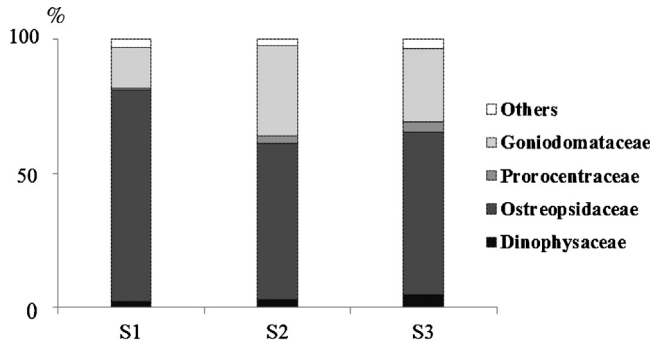

Fig. 2. Relative abundance of dominant dinoflagellate groups in the Punic harbors.

2009 were registered at S2 which is situated in the arc-shaped north basin. The lowest salinity (33.50) was recorded at both S1 and S3 in May, with the highest (39) observed in September 2009 (Table 1).

3.2. Dinoflagellate composition and dynamics

Analysis of the relative abundances of the dominant orders of dinoflagellates at different sampling stations shows that Ostreopsidaceae are the most abundant, accounting for 30–40% of the total (Fig. 2). The Goniodomataceae represent 20% at S2, but only 8% at S1 and 15% at S3. The Dinophysaceae and Prorocentraceae are less abundant (2–4%) (Fig. 2).

Despite the relatively low abundance of species belonging to the order of dinophysiales five species belonging to the genus *Dinophysis* were identified during this study, namely *Dinophysis acuminata*, *Dinophysis cf. ovum* Schütt, *Dinophysis sacculus* Stein, *Dinophysis rotundata* Claparède and Lachmann, and *Dinophysis caudata* Saville-Kent.

During our survey in the Punic harbors of Carthage, *Dinophysis sacculus* exhibited different morphotypes as described by Stein (1883): one with a slender rectangular form (Fig. 3H), the second almost sack-like (Fig. 3I), another more slender and rectangular and the last with a mid-dorsal concavity (Fig. 3G). Cell length varied from 48 to 52 μm and the width from 26 to 32 mm.

Cells of *Dinophysis cf. ovum* had an ellipsoid form, widest near the middle part of the cell with a more or less small cingular list (Fig. 3A–C). Cell size varied from 42 to 48 μm in length (44.52 ± 2.52, $n = 100$) and 27 to 37 μm in width (31.62 ± 2.29, $n = 100$) with a length–width ratio (L/W) ranging from 1.20 to 1.50 (1.41 ± 0.17, $n = 100$). *Dinophysis acuminata* presented cells with characteristics fairly close and similar to its original description (Fig. 3D–F) (Claparède and Lachmann, 1858, 1859). Cell length varied from 44 to 52 μm and the width from 25 to 36 mm.

Microscopic identification of *Dinophysis rotundata* and *Dinophysis caudata* is easy owing to their morphological characteristics. Indeed, cells of *D. rotundata* are medium-sized and broadly rounded in the lateral view with convex ventral and dorsal margins (Fig. 3J). *D. caudata* is a very distinctive species: large, long and irregularly subovate with a long ventral projection on the hypotheca (Fig. 3K and M).

Dinophysis acuminata and *Dinophysis sacculus* showed a wide temporal distribution throughout the sampling period.

No significant difference in *Dinophysis* abundances was found among sampling sites (RDA, $F = 0.71$; $p = 0.62$), however a significant change was seen among the seasons (RDA, $F = 44.05$, $p < 0.001$). This temporal variability explains 27.41% of changes in *Dinophysis* abundances with RDA axes 1 and 2 both supporting a significant effect on this variability ($p < 0.05$) (Figs. 4 and 5). Along RDA axis 1, *Dinophysis cf. ovum* stands out with a negative score and is thus related to spring observations. Along RDA axis 2, the segregation observed in summer was associated with *Dinophysis sacculus*, *Dinophysis rotundata* and *Dinophysis caudata*, *Dinophysis acuminata* appears to be associated with spring, winter and autumn.

Dinophysis acuminata occurred in water samples throughout the year, with its highest densities recorded during June 2009 (6.7×10^3 cells l^{-1}). Other important proliferations were observed from October to November (from 10^3 to 6×10^3 cells l^{-1}, 2009), but the species was absent during July and August (Fig. 6). *Dinophysis cf. ovum* occurred in water samples from January to early June. High concentrations (1.90×10^4 to 6.30×10^4 cells l^{-1}) were recorded from March to June (2008–2009) (Fig. 6). *Dinophysis sacculus* occurred in the Punic harbors in significant concentrations (3×10^3 to 2.25×10^4 cells l^{-1}) from May to July (2008–2009), low to moderate from September to November (2008–2009; 70–2.10×10^3 cells l^{-1}) and sporadically during the rest of the year (Fig. 6).

3.3. In situ *division rate* (μ)

Table 2 gives the estimated minimum division rate μ_{min} (Vaulot, 1992), for *Dinophysis acuminata* and *Dinophysis sacculus* from each studied cycle, and the mean daily specific μ, T_c, and T_r using cytokinesis and sulcal list regeneration as terminal events (μf_{c+r}). The data is adjusted to a Gaussian function as described in Reguera et al. (2003) and Velo-Suárez et al. (2009) after the model of Carpenter and Chang (1988).

During the early summer cycles, *Dinophysis acuminata* exhibited moderate μ (0.22–0.39 day^{-1}) and μ_{min} of (0.16–0.22 day^{-1}). The highest value of the daily average specific division rate (0.68 day^{-1}) was recorded at S3; however, this was less than half the rate found at the other stations (0.22–0.50 day^{-1}). In contrast, low μ_{min} (0.10–0.16 day^{-1}) values and moderate mean daily specific μ (0.21–0.35 day^{-1}) of *Dinophysis sacculus* were characteristic of this diel cycle.

Low estimated minimum growth rates (μ_{min}) (0.12–0.15 day^{-1}) and moderate mean daily specific μ (0.20–0.39 day^{-1}) 1) of this species were recorded at different stations during the autumn diel cycle while in the same cycle very low minimum division rates of *Dinophysis sacculus* (0.16–0.19 day^{-1}) were estimated at S2 and S3.

3.4. Cell deformations

10–20% of cells of *Dinophysis acuminata*, *Dinophysis cf. ovum* and *Dinophysis sacculus* were observed during their cell cycle with deformations at their ventral and dorsal margin. Thus, it was not possible to clearly distinguish between *D. acuminata* and *D.* cf *ovum* and we therefore assumed that the cells were of *D. acuminata*. In addition, during summer and autumn diel cycles, the study of relative abundances and frequencies showed that *D. acuminata* and *D. sacculus* represented 80% of the other dinophysiales species. Since dorsal and ventral margin deformations did not allow us to distinguish between cells of *D. acuminata* and *D. cf. ovum*, we used the nomenclature "*D. acuminata*/*D. cf. ovum* complex".

Two major types of cell deformation were observed:

- *Dinophysis* cells with invaginations and protuberances in the ventral margin:

  Two different invagination shapes were observed at the base of the left sulcal list of the cell: one in the shape of an arc (Fig. 7A), the other, smaller, but markedly concave (Fig. 7B). Other cells showed small and moderate invaginations in the left part of the antapical region (Fig. 7C).

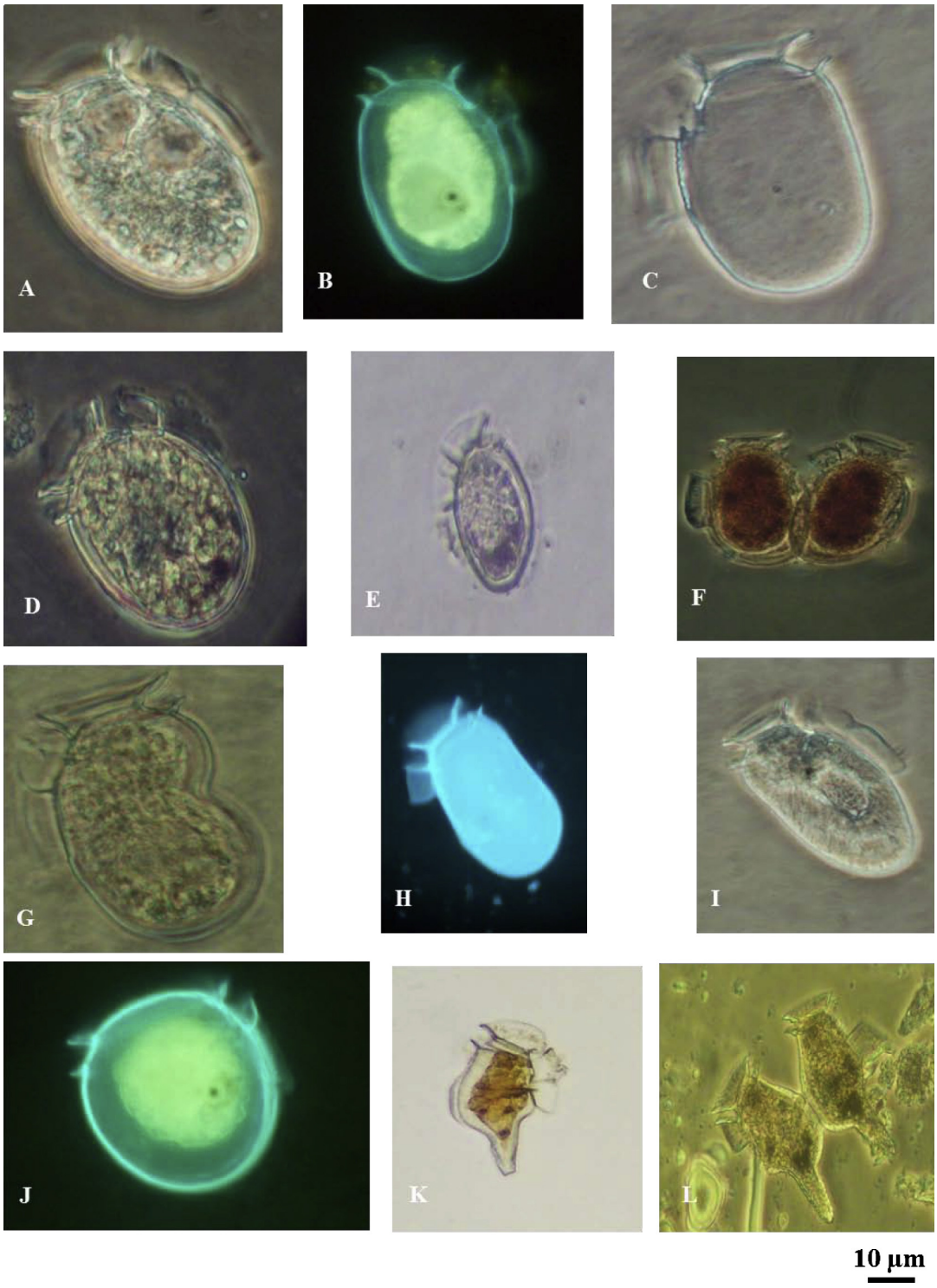

Fig. 3. *Dinophysis* species found in the Punic harbors of Carthage from March 2008 to June 2010. (A–C) *D. cf. ovum* from spring sampling. (A) Fully developed cell in light microscope. (B) DAPI-stained cell. (C) Theca of recently divided cell showing the posterior half of the left sulcal list. (D–F) *D. acuminata* from summer sampling. (D and E) Fully developed cell in light microscope. (F) Paired cells. (G–I) *D. sacculus*. (G) Fully developed vegetative cell. (H) DAPI-stained recently divided cell showing the anterior half of the left sulcal list. (J) DAPI-stained cell of *D. rotundata*. (K) Fully developed cell of *D. caudata*. (L) Recently divided pair of *D. caudata*.

The other shapes of deformations were the protuberances observed at the base of the left sulcal list of *Dinophysis* cells (Fig. 7D). After dissection, the protrusion, having a spherical oval shape, was visible in both valves of the theca, though clearer on the right. Fig. 7E and F shows that areolation is uniform on the left valve, including the protuberance.

- *Dinophysis* cells with invaginations in the dorsal margin:

All invaginations observed in both recently divided and fully developed cells of *Dinophysis acuminata*/*Dinophysis cf. ovum* complex were observed in the posterior part of the dorsal margin (Fig. 7H–J).

Slight or more marked mi-dorsal concavity is well known in *Dinophysis sacculus* cells (Stein, 1883). Nevertheless, introversions in the posterior part of the dorsal margin were observed in

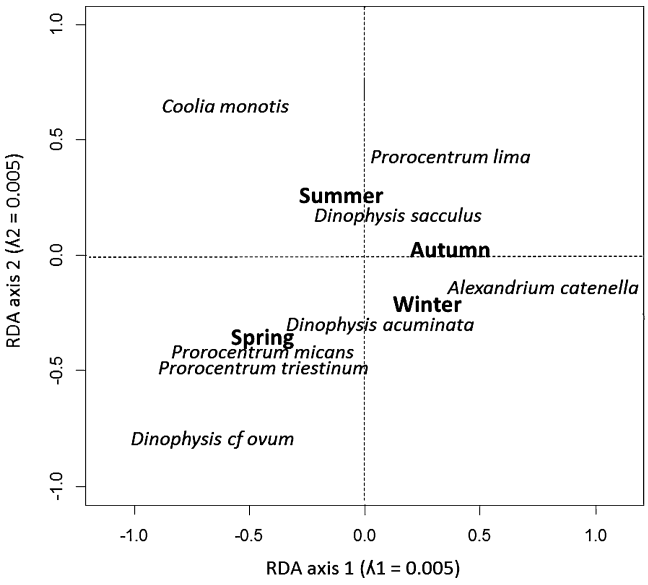

Fig. 4. RDA TriPlot depicting the association between dominant dinoflagellate species and sampling sites. Eigenvalues of the first two axes are indicated by λ1 and λ2.

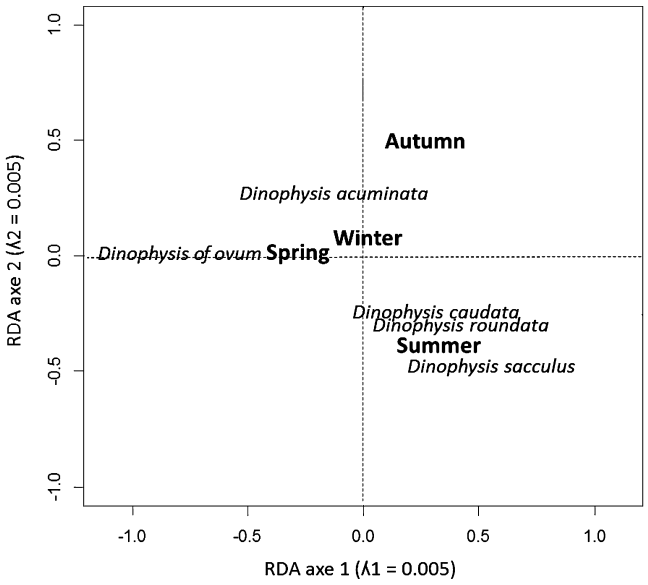

Fig. 5. RDA TriPlot depicting the association between *Dinophysis* species and sampling seasons. Eigenvalues of the first two axes are indicated by λ1 and λ2.

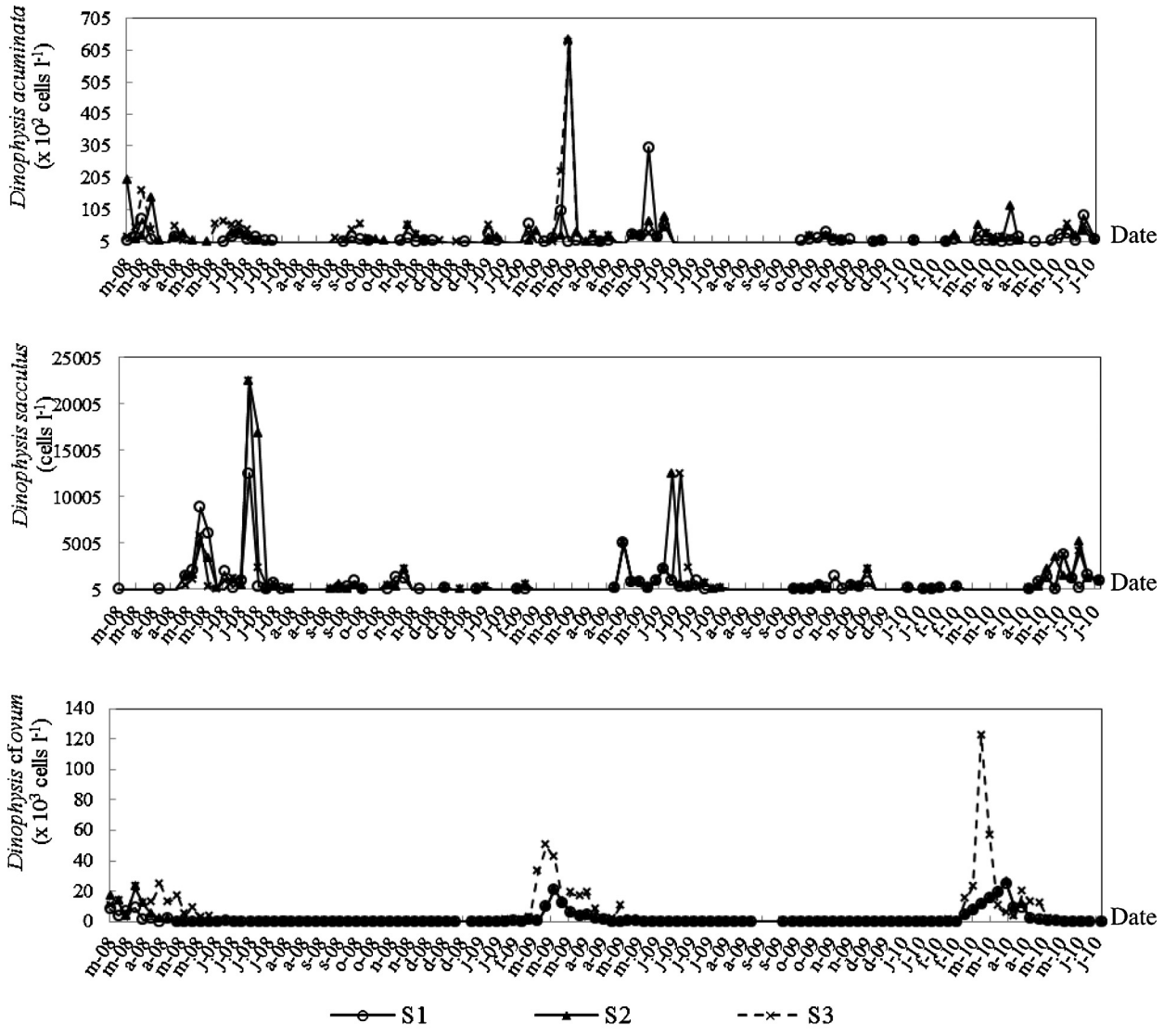

Fig. 6. Spatiotemporal variations of *Dinophysis* species from March 2008 to June 2010 in the Punic harbors.

Table 2
In situ estimated division rates (mitotic index approach; day^{-1}) for *D. acuminata* and *D. sacculus*.

Species	Location	Date	μ (day^{-1})	μ_{min} (day^{-1})	Sources
D. acuminata	Long Island, NY, USA	July 1997	0.54		Chang and Carpenter (1991)
	Galician Rías Baixas, NW, Spain	1–2 June 1994	0.28–0.46 ± 0.05	0.25	Reguera et al. (2003) Velo-Suárez et al. (2009)
		27–28 October 1994	0.26	0.13	Reguera et al. (2003)
		15–16 June 1994	0.09	0.08	
		June 2005	0.56		González-Gil et al. (2010)
	Gullmar Fjord, Sweden	October 1995	0.75		Gisselson et al. (1999)
	Punic harbors of Carthage, Gulf of Tunis, Tunisia	3–4 June 2008			Present paper
		S1	0.50	0.13	
		S2	0.33	0.13	
		S3	0.30	0.21	
		2–3 November 2008			
		S1	0.39	0.12	
		S2	0.21	0.14	
		S3	0.20	0.15	
		15–16 June 2009			
		S1	0.33	0.21	
		S2	0.37	0.22	
		S3	0.68	0.21	
		30–31 October 2009			
		S1	0.16	0.14	
		S2	0.25	0.13	
		S3	0.33	0.15	
		26–27 June 2010			
		S1	0.27	0.16	
		S2	0.22	0.17	
		S3	0.39	0.22	
D. sacculus	Ebro Delta, Spain	May 1994	0.42		Garcés et al. (1997)
		June 1994	0.28		
		June 1994	0.38		
		October 1994	0.2		
	Punic harbors of Carthage, Gulf of Tunis, Tunisia	3–4 June 2008			Present paper
		S1	0.09	0.04	
		S2	0.23	0.18	
		S3	0.26	0.21	
		2–3 November 2008			
		S1	0.09	0.02	
		S2	0.16	0.12	
		S3	0.19	0.16	
		15–16 June 2009			
		S1	0.10	0.04	
		S2	0.21	0.21	
		S3	0.23	0.21	
		30–31 October 2009			
		S1	0.16	0.10	
		S2	0.17	0.11	
		S3	0.14	0.10	
		26–27 June 2010			
		S1	0.21	0.16	
		S2	0.35	0.15	
		S3	0.32	0.10	

D. sacculus and *Dinophysis acuminata*/*Dinophysis cf. ovum* complex cells.

Fig. 7I and J shows fully developed cells with dorsal and ventral invaginations, though we cannot distinguish the left sulcal list of the severely deformed *Dinophysis* cell (Fig. 7M). These latter observations were very rare during diel cycle sampling.

4. Discussion

Dinophysis blooms are initiated when abundance is $>10^2$ cells l^{-1}, as observed by quantitative methods (Reguera et al., 2012, 2014). According to Margalef et al. (1979), *Dinophysis* species, like other dinoflagellates, are favored by the relative absence of turbulence, leading to their development in warm, stratified waters, from late spring to early autumn (Smayda, 1980). This is consistent with the high numbers of *Dinophysis acuminata* and *Dinophysis caudata* found in the Ria of Vigo and Pontevedra during early summer and autumn (Reguera et al., 2003). However, other studies have reported different *Dinophysis* species ranging from very low (<20 cells l^{-1}) (Bravo et al., 1995) to high concentrations ($>85.4 \times 10^3$ cells l^{-1}) (Koukaras and Nikolaidis, 2004) throughout winter. Many other studies have highlighted the origin of *Dinophysis* populations in different areas, but no study shows how the species survives through environmentally disadvantageous circumstances such as those existing in the winter months in temperate regions (Reguera et al., 2012). As they do in all the coastal Mediterranean waters (Caroppo, 2001; Koukaras and Nikolaidis, 2004; Ninčević-Gladan et al., 2008), *D. acminata* and *Dinophysis sacculus* start to increase in the Punic harbors when the water column is stable, from March to June and from October to November, whereas *Dinophysis cf. ovum* proliferate from the beginning of January to the end of June. According to Maestrini (1998) and Aubry et al. (2000) *Dinophysis*

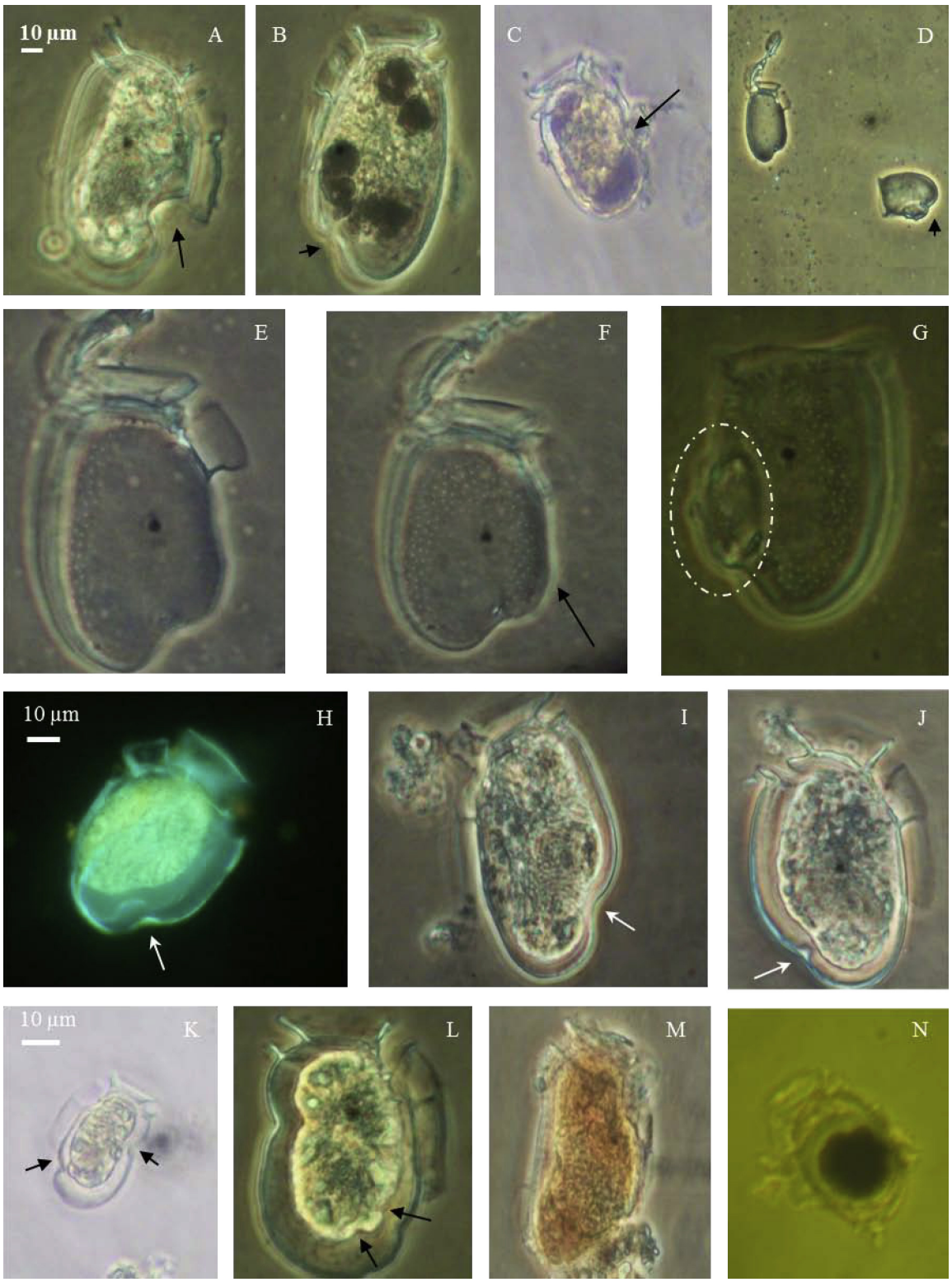

Fig. 7. Invaginations and protuberances in the dorsal and ventral *Dinophysis* cells from the Punic harbors. (A) *D. cf. acuminata* with strong deformation in the ventral margin, at the base of the LSL. (B) *D. cf. acuminata* with deformation in the left antapical region. (C) *D. cf. acuminata* with small deformation in the ventral margin, by the base of the LSL. (D) Dissection of cells with protuberances on the base of the LSL. (E) Lateral view (theca) of a recently divided cell (after dissection) showing a protuberance on the left part of dorsal margin. (F) The theca shows a uniform areolation, including the protuberance. (G) Side view of the theca of a recently divided cell showing a protuberance. (H) Posterior dorsal margin invagination of a recently divided cell of *D. acuminata* (DAPI stained). (I) Strong posterior dorsal margin invagination in a fully developed cell of *D. cf. acuminata*. (J) Other type of posterior dorsal margin of a fully developed cell of *D. cf. acuminata*. (K) One invagination in the base of the left sulcal list of a cell of *D. sacculus*. (L) Two small invaginations in the posterior part of the ventral margin and one in the anterior ventral margin. (M) Very deformed cell of *D. sacculus*. (N) Infected cell of *D. acuminata* (round disc).

species represent only a fraction of total phytoplankton assemblages, a conclusion largely confirmed in our study (2–4% of total dinoflagellates).

Redundancy analysis shows that this species occurred in high abundance in spring, with high densities of *Dinophysis sacculus* and *Dinophysis acuminata* observed in summer and autumn. The proliferation of *Dinophysis* species within extreme ranges of temperature (11.6 °C in February 2008, 30 °C in August and September 2009) and salinity (40.30 in August 2008 and 32 in December 2009) demonstrates their tolerance for wide variations of these parameters, but they also require specific values which appear to stimulate their growth.

During autumn cycles, values of μ_{min} were close to those of μ_{fc+r}, especially for *Dinophysis sacculus*. This species shows high μ_{min} values (0.21 day^{-1}) in summer cycles (June 15th and 16th, 2009), and low values in autumn cycles (Table 2). In agreement with our observations, Garcés et al. (1997) report that *in situ* estimated division rates by mitotic index approach of *D. sacculus* were higher in early summer and lower in autumn. *D. sacculus* was observed in the Punic harbors with moderate μ_{min} estimated in the June sampling cycle and an increase in its population was observed the following day. After a high tide occurring during the night, water flowing from the Gulf of Tunis into the basins of both harbors may contain high concentrations of *D. sacculus* cells which, on arriving inland from the gulf, may divide rapidly. In fact, the coastal *Dinophysis* species appear to be excellent survivors, able to modulate their division rates, adopting extremely low rates to persist in the water column in the absence of prey, which is why they should not be considered as slow-growers (Reguera et al., 2012). This may explain the presence of this species throughout the year in the Punic harbors. Velo-Suárez et al. (2009) suggested that *Dinophysis acuminata* sampled during the June cycle divided at the same rate throughout the entire water column, and that the high concentrations of this species observed at different depths were caused by vertical migration and advection and not by higher division rates. This suggestion was largely confirmed in our study; indeed, higher values of daily average specific division rate were obtained for *D. cf. acuminata* (Table 2). It may be that the shallow depths of the Punic harbors have no influence on the estimates of *in situ* division rates of *D. acuminata*.

Dinophysis acuminata and *Dinophysis sacculus* bloomed in the same period and/or simultaneously and appeared to have different strategies and patterns of cell division. This may be explained by their different ecological behavior; many studies (Jacobson and Anderson, 1996; Janson, 2004; Park et al., 2006) have shown that *D. acuminata* is an obligate mixotroph. Caroppo (2001) proposed that *D. sacculus* in the Mediterranean Sea (Varano Lagoon) might be autotrophic but recently, *D. sacculus* from the Galician Rías was established in mixotrophic cultures with *Mesodinium rubrum*as prey (Riobó et al., 2013).

Deformations at the ventral and dorsal margin were observed in both recently divided and/or fully developed cells of *Dinophysis* species complexes in samplings of different diel cycles. As far as we know, this is the first time ever that these deformations, in the form of invaginations and protuberances at the dorsal and ventral margins, have been seen. The origin of these strains is unknown, but in the light of different previous observations, especially those concerning polymorphic sexual cycles (MacKenzie, 1992; Bardouil et al., 1991; Moita and Sampayo, 1993; Reguera et al., 1995; Giacobbe and Gangemi, 1997; McLachlan, 1993; Reguera and González-Gil, 2001; Escalera and Reguera, 2008), two hypotheses can be proposed:

- Cell deformations may be encountered during the various stages of the *Dinophysis* sexual cycle.

  The *Dinophysis* sexual cycle has been studied by many authors: Hansen (1993) and Reguera et al. (1995) observed couplets between normal-sized and small cells attached by their ventral margins, the smaller cell being engulfed by the larger one. Pairs of cells united by the dorsal margins are thought to be the result of vegetative division. Koike et al. (2006) suggested that the larger cell engulfs the smaller one through the cingulum during cell fusion. Reguera and González-Gil (2001) suggested that small cells of *Dinophysis* spp. may become normal-sized cells if not involved in planozygote formation. Planozygote forms were observed in natural populations of *Dinophysis acuminata* throughout their entire growing season (Gentien et al., 2004). According to some recent observations, planozygotes may be able to divide with no need to go through hypnozygote formation (Escalera and Reguera, 2008). The result of the first meiotic division was assumed to be a tetrad by Reguera and González-Gil (2001). This form has been observed in different cultures of *Dinophysis* spp. (Nagai et al., 2008; Nishitani et al., 2008a,b) and described as "sequential binary fission". The *Dinophysis* cells with invaginations and protuberances at the dorsal and ventral margin that were observed in the Punic harbors had never before been recorded in studies of the *Dinophysis* species life cycle. Despite the successful subsequent creation of *Dinophysis* cultures, no new observations concerning the species' life cycle have been reported (Reguera et al., 2012).
- Invaginations and protuberances observed at the ventral and dorsal margins of *Dinophysis* cells resulted from infection by parasites, viruses or bacteria.

  As hosts, dinoflagellates contain viruses, bacteria, fungi, and other protists of which athecate species are known to be the most infected (Park et al., 2004). Found in dinoflagellate cytoplasm, these organisms are in the form of round discs (Canter, 1961, 1968); those detected in *Dinophysis acuminata* were later interpreted as sporangia of a *Parvilucifera*-like parasite (Norén et al., 1999). We can therefore conclude that dinoflagellates may be infected by parasites, viruses and bacteria, some of which can kill them without changing their shapes. The cell deformations at the dorsal and ventral margins of *Dinophysis* spp. observed in the Carthage Punic harbors do not appear to have originated as viral infections. Indeed, *D. acuminata* cells, observed outside of diel cycle sampling and having a large round disc (Fig. 7N) may be infected by a parasite.

5. Conclusion

Our field observations in the Punic harbors of Carthage show that *Dinophysis cf.* ovum, *Dinophysis acuminata* and *Dinophysis sacculus* are found under distinct environmental conditions, indicating that these species tolerate wide variations in water temperature and salinity. RDA analysis showed greater proliferation of *D. cf. ovum* in spring, whereas *D. acuminata* and *D. sacculus* blooms were abundant in summer.

These different species require specific values which appear to stimulate their growth. *Dinophysis acuminata* and *Dinophysis sacculus* can exhibit a wide range of values in their intrinsic division rates, between practically 0 and 0.68 day^{-1}.

Invaginations and protuberances have been observed at the dorsal and ventral borders of recently divided and fully developed cells of *Dinophysis* species recorded for the first time in the world's oceans and seas. Further prospective molecular and genetic studies and staining with specific fluorescent strains may hopefully explain these *Dinophysis* cell deformations during their *in situ* division.

Acknowledgments

This work is part of a collaborative project between the INSTM (Institut National des Sciences et Technologies de la Mer, Tunisia),

the University of Franche-Comté (Chrono-Environnement, UMR CNRS 4269, Besançon, France) and the Instituto Español de Oceanografia (IEO), Centro Oceanográfico de Vigo, Spain. We are grateful to the members of IEO and CNRS 4269 for their kindness and help. We also thank Dr. Lourdes Velo-Suárez (IFREMER, Centre de Brest) for her helpful comments and advice during this study. We thank Radhia Souga for her valuable help during the sampling work. We express our appreciation to the Editor Dr. Sandra E. Shumway and the anonymous reviewers for helping to improve our paper.[SS]

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